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# *Cryptosporidium parvum* oocyst inactivation in three soil types at various temperatures and water potentials

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**Abstract**

The interaction between soil types, temperature, and soil water potential may have differential effects on the survival of *Cryptosporidium parvum* oocysts in the terrestrial environment. We examined the effects of three soil types (a silty clay loam, silt loam, and loamy sand), three temperatures (4, 20, and 30 °C), and three soil water potentials (−0.033, −0.5 and −1.5 MPa) on the inactivation kinetics of oocysts. Sentinel chambers were filled with air-dried and sieved soil, brought to the appropriate soil water potential, and inoculated with $2 \times 10^6$ freshly purified oocysts. The inoculated chambers were buried in the same bulk soil at the appropriate water potentials and incubated at one of the three temperatures. Triplicate chambers were removed from the bulk soil on days 0, 22, 43, 84 and 156. Sentinel oocysts were extracted, and assayed for potential infectivity by the dye permeability method. Oocysts suspended in sterile distilled water and incubated with the sentinel chambers were used as controls for the effect of temperature. The soil water potentials investigated did not affect oocyst inactivation at any temperature or with any of the three soil types. Rates of oocyst inactivation increased significantly between 4 and 20 °C, but not between 20 and 30 °C with the exception of oocysts incubated in the silty clay loam. Oocyst survival appeared to be significantly greater in the silt loam soil than in the two other soil types when incubated at 20 °C; and at 30 °C oocyst survival was significantly less in the silt clay loam than in the other two soil types. Rates of sentinel oocyst inactivation at all three soil water potentials were significantly lower than the control oocysts in water at the three test temperatures. Thus oocyst survival in soil was not affected by the water potentials between −0.033 and −1.5 MPa; it was affected by soil texture; but temperature appeared to be the factor most affecting oocyst survival. In the critical ambient range of temperature in temperate climates oocysts may survive for months in agricultural soil, and pose a threat to surface waters. 

*Keywords*: *Cryptosporidium parvum*; Oocysts; Inactivation; Soil type; Water potential; Temperature

## 1. Introduction

In watersheds both point and non-point sources contribute to the load of pathogenic microorganisms. Point sources such as wastewater treatment plants are known sources of pathogens (Rose, 1997). Where animal agriculture is extensive within a watershed, non-point sources of pathogens have become a focus of attention. The protozoan parasite *Cryptosporidium parvum* is an emerging zoonotic pathogen that poses a critical public health problem, and has become a particular concern to the water industry (Berkelman, 1994; Rose, 1997) world-wide since it caused a massive outbreak of gastrointestinal illness in Milwaukee, WI in 1993 (MacKenzie et al., 1994) as well as significant outbreaks of cryptosporidiosis in the United Kingdom and Japan (Smith and Rose, 1998). Amendments to the Safe Drinking Water Act in 1996 that required managers of public water supplies to identify potential sources of contamination, and the US Environmental Protection Agency's establishment of guidelines to carry out local source water assessments (Walker et al., 1998) increased research efforts in the US on *C. parvum* in the environment.

*C. parvum* is associated with feces of humans, cattle, and wild mammals in woodlands (Fayer, 1997). Previous studies indicated that there was a strong positive correlation between *C. parvum* loads in watersheds and the presence of cattle (Hansen and Ongerth, 1991; LeChevallier et al., 1991) and calving activities (Ong et al., 1996). *C. parvum* has been the cause of several outbreaks of gastrointestinal illness associated not only with municipal drinking water systems but also with swimming in lakes, and ground water (Rose et al., 1991; Rose, 1997; Hancock et al., 1998; Centers for Disease Control, 2000).

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Two genotypes of *C. parvum* have been identified, Type I observed in human sources, and Type II observed in bovine sources (Peng et al., 1997). Type I genotypes do not infect mice or calves and appear to be specific to humans. Because Type II genotypes are zoonotic, cattle are considered an important reservoir of human infection in the terrestrial environment (Mawdsley et al., 1995; Atwill, 1996). Neonatal calves, in particular, can shed billions of oocysts during the period of initial infection (Anderson, 1981; O'Handley et al., 1999).

Little is known about the survival of *C. parvum* oocysts in terrestrial environments once they are shed by their host (Hansen and Ongerth, 1991; Robertson et al., 1992). Factors affecting survival and causing relatively rapid inactivation of *C. parvum* oocysts are extreme temperatures ($< -10$ and $>35$ °C) (Fayer, 1994; Fayer and Nerad, 1996; Jenkins et al., 1997), extreme pH such as pH 1 and 13 (Campbell et al., 1992), desiccation (Anderson, 1986; Walker et al., 1998) and ammonia (Jenkins et al., 1998).

Freire-Santos et al. (2000) reported that 20% of oocysts exposed to a salinity of 35‰ at 18 °C survived for 40 days. Several studies have indicated that *C. parvum* oocysts can survive in raw waters for extended periods (Robertson et al., 1992; Johnson et al., 1997; Medema et al., 1997). Chauret et al. (1998) showed that oocysts aged in raw water were as resistant to chlorine disinfection as unaged oocysts indicating that exposure to raw, natural waters under ambient water temperatures has an insignificant effect on oocyst inactivation.

Olson et al. (1999) demonstrated that purified oocysts were able to survive longer than 12 weeks in water, normal soil, and autoclaved soil at 4 °C; but at 25 °C inactivation occurred at 12 weeks. They also reported that nearly total inactivation of oocysts incubated in cattle feces at 4 and 25 °C occurred by 12 weeks. In contrast, Jenkins et al. (1997) reported that 10% of oocysts in naturally infected feces survived longer than 400 days at 4 °C. Jenkins et al. (1999) reported that *C. parvum* oocysts in surface soil under field conditions survived for weeks with negligible inactivation at temperatures near freezing and field capacity until freeze–thaw cycles appeared to inactivate them.

An understanding of how long oocysts can survive in soil would enable managers of municipal watersheds in which agriculture occurs (e.g. New York City watershed) to improve best management practices and better assess the risk of *C. parvum* contamination of drinking water. Our objective was to determine if variations in soil water potential and temperature affected *C. parvum* oocyst rates of inactivation in three distinct soil types, and estimate durations of oocyst survival under controlled laboratory conditions. Four null hypotheses and their alternatives were tested. (1) $H_O$: rates of oocyst inactivation for each soil type do not differ between the three soil water potentials at the three incubation temperatures; $H_A$: the rates of oocyst inactivation differ between the soil water potentials tested. (2) $H_O$: rates of oocyst inactivation for each soil type will not differ between the three incubation temperatures; $H_A$: the rates of oocyst inactivation differ between the three incubation temperatures. (3) $H_O$: rates of oocyst inactivation do not differ between soil types; $H_A$: the rates of oocyst inactivation differ between soil types. (4) $H_O$: rates of oocyst inactivation associated with the soil treatments do not differ from the temperature controls or oocysts in distilled water; $H_A$: the rates of oocyst inactivation for soil treatments differ from temperature control oocysts in water.

## 2. Materials and methods

### 2.1. C. parvum oocysts

*C. parvum* oocysts were obtained from naturally infected, 7- to 14-days-old calves in Tompkins County, NY. A sucrose-Percoll™ flotation method was used to extract oocysts from calf feces (Jenkins et al., 1997). After extraction, oocysts were stored at 4 °C in water with antibiotics (100 U of penicillin G sodium $ml^{-1}$, 100 μg of streptomycin sulfate $ml^{-1}$, and 0.25 μg of amphotericin B $ml^{-1}$), and were used within 2 weeks of collection. Concentrations of stock oocyst suspensions were enumerated with a hemacytometer before dilutions were made for inoculation. An inoculant suspension of $5 \times 10^7$ oocysts $ml^{-1}$ was determined by replicate counts using a hemacytometer. Viability as determined by the dye permeability assay (as described later) was made concurrent with concentration determinations. At the time of use viability ranged from 90 to 95%.

### 2.2. Soils

The three soil types used in this study were from field sites within the New York City watershed. They differed in their textural characteristics, and based on their particle size distribution (Shirazi et al., 1988) were classified as a silty clay loam, silt loam, and loamy sand. The silty clay loam was a fine-silty, mixed, mesic, aquic glossudalfs belonging to the Collamer soil series; the silt loam was a coarse, mixed, frigid, typic fragiudepts belonging to the Willowmoc soil series; and the loamy sand was a course-loamy, mixed, mesic, typic dystrudepts belonging to the Riverhead soil series. Physical and chemical characteristics of these soils were determined by the Soils Testing Laboratory, Department of Soils, Crops, and Atmospheric Sciences, Cornell University and are listed in Table 1. Moisture release curves were established for each soil type by the Soils Testing Laboratory at Cornell University, and the quantities of water needed to obtain soil water potentials of $-0.033$, $-0.5$, and $-1.5$ MPa were based on the linear regression equation for each curve where gravimetric water content was regressed against the log of the pressure plate measurements. The soils were air-dried at 37 °C, and sieved (2 mm). Air-dried and sieved soils were stored in a lidded bucket at 4 °C until used.

Table 1
Physical and chemical characteristics of the three soil types used in experiments

| Characteristics | Silty clay loam (%) | Silt loam (%) | Loamy sand (%) |
|---|---|---|---|
| Total sand | 1.8 | 33.4 | 80.3 |
| Total silt | 68.8 | 50.3 | 14.3 |
| Total clay | 29.4 | 16.3 | 5.0 |
| Organic C | 2.63 | 2.29 | 1.8 |
| | Silty clay loam (cmol $kg^{-1}$) | Silt loam (cmol $kg^{-1}$) | Loamy sand (cmol $kg^{-1}$) |
| CEC | 18.37 | 17.17 | 7.10 |
| Ca | 6.78 | 8.64 | 3.99 |
| Mg | 0.98 | 1.66 | 0.41 |
| K | 0.09 | 0.43 | 0.21 |
| Na | 0.02 | 0.04 | 0.00 |
| pH | 4.60 | 7.10 | 5.48 |

### 2.3. Containers, sentinel chambers and experimental design

The soils were held in disposable plastic containers (9.5 cm diameter, 5.5 cm tall) with plastic lids. Each container was filled with 230 g of the air-dried soils. In replicates of three, each soil type was brought to one of the three water potentials, −0.003, −0.5, and −1.5 MPa, and allowed to equilibrate for several days. The water contents of the containers were monitored gravimetrically, and water was added as needed.

The sentinel chamber method developed by Jenkins et al. (1999, 2000) was used to monitor oocyst viability in the soils. The sentinel chamber consisted of the basket of a commercially available microfiltration system (2.5 cm long, internal diameter 0.7 cm, Osmonics, Livermore, CA) with a nylon 0.45 μm-pore-size filter encased at one end; at the other end the cap of the system was perforated and used to secure a 60 μm nylon mesh filter (SpectraMesh, Markson, Inc., Hillsboro, OR). The chamber was designed to contain a representative soil sample, to equilibrate with the solute and moisture regime of the external environment, and to be easily retrievable and processed for analysis. Based on the weight of the air-dried soil put in the chambers (approximately 0.9 g of silt loam and loamy sand soils, and 0.6 g of the silty clay loam) a calculated amount of water (minus the inoculum volume of 40 μl) was added with a syringe to bring the soils to one of the three water potentials. Five chambers per container were then embedded in bulk soil according to their water potential and allowed to equilibrate for 2 days. After equilibration the chambers were removed from the bulk soil and inoculated with $2 \times 10^6$ oocysts suspended in 40 μl of water using a 100 μl Hamilton Syringe. The oocysts were dispersed in the sentinel chamber by moving the syringe needle from near the bottom to near the top as the oocysts were injected. The inoculated sentinel chambers were reinserted into their respective replicate bulk soil containers. Each bulk soil container was then placed as a completely randomized block in separate incubators set at 4, 20 or 30 °C.

As controls, $1 \times 10^6$ oocysts were suspended in sterile distilled water in microcentrifuge tubes. The control oocysts were incubated with the soil containers. Thus the inactivation of the control oocysts was affected by temperature only; comparison of the soil treatments with the controls allowed the effects of the soil matrix on oocyst inactivation to be discerned at each temperature and water potential tested.

### 2.4. Oocyst extraction from chambers

One sentinel chamber was removed from each replicate soil container on days 1, 22, 43, 84 and 156 along with three controls. Oocysts were then extracted from the contents of the sentinel chamber and with control oocysts were assayed for potential infectivity using the dye permeability assay. The entire quantity of soil was removed from the chambers and put into a 15 ml polypropylene centrifuge tube containing 7 ml of a 0.5% 7X detergent (Linbro 7×, Thomas Scientific, Swedesboro, NJ) in phosphate buffered saline (PBS). A small amount of Zirconia/silica beads (0.5 mm) (BioSPec Products, Inc., Bartlesville, OK) were added to facilitate dislodging sorbed oocysts, and the soil, beads, and detergent were mixed for 20 s using a vortex mixer. The 7 ml mixture was then underlaid with 7 ml of cold sugar solution (specific gravity 1.18). After the samples were centrifuged at $1800 \times g$ for 20 min at 4 °C, about 3 ml of the interface was gently removed and transferred to a second tube. Distilled water, 12 ml, was added to the sample; the diluted sample was mixed thoroughly and centrifuged again at $1800 \times g$ for 20 min at 4 °C. The supernatant was aspirated leaving approximately 1 ml into which the pellet was resuspended, transferred to a microcentrifuge tube, and sedimented at $11{,}000 \times g$ for 5 min. The supernatant was then removed by aspiration, and the pellet was resuspended in 100 μl PBS in preparation for the dye permeability assay. Extraction efficiency for the silt loam soil held at 25 °C and −0.033 MPa ranged from 50 to 90%; extraction efficiencies for the same field soil exposed to freezing temperatures and soil water potentials as negative

as −3.6 and −7.7 MPa ranged from 0.1 to 0.6% (Jenkins et al., 1999). The large concentration of inoculum was comparable to soil on which a calf with cryptosporidiosis had defecated (O'Handley et al., 1999). The large inoculum was meant to facilitate counting oocysts from extracted samples and to provide sufficient oocysts from samples with low percentage extraction efficiencies. The objective of this inoculation protocol was to assay percent viability of 100 oocysts representative of the population per sample. Obtaining a mass balance of oocysts per sample was not an objective.

For each assay duplicate 10 μl subsamples of each replicate were placed on an agar-coated microscope slide and a $22 \times 22$ mm$^2$ cover slip was applied with slight pressure to spread the sample evenly to the edge of the cover slip (Jenkins et al., 1997). At least 100 oocysts in randomly chosen fields in each smear were identified by immunofluorescence and categorized by differential dye uptake by epifluorescence and structure by DIC microscopy (Anguish and Ghiorse, 1997). Values for each replicate were the mean of the duplicate subsamples.

### 2.5. Dye permeability staining

This assay has been described by Campbell et al. (1992), Anguish and Ghiorse (1997) and Jenkins et al. (1997). Further details of its applicability as an indicator of viability or potential for animal infectivity and thus the capability of reproduction under appropriate conditions were discussed by Robertson et al. (1998). As explained in the reply to Robertson et al. (1998), to assay the viability of oocysts extracted from an environmental matrix such as soil the dye permeability assay is treated explicitly as an indirect assessment of potential infectivity. As such, this dye permeability method (Jenkins et al., 1997) was adapted to USEPA's method 1622 in a study assessing viability of oocysts extracted from surface waters (Simmons et al., 2001). Furthermore, Jenkins et al. (Robertson et al., 1998) explained that oocysts impermeable to 2-4′diamidino-2-phenylindole (DAPI) and propidium iodide (PI), and showed internal structure such as the outline of sporozoites and residual body under differential interference contrast (DIC) microscopy, were viable as demonstrated by animal infectivity assays (Jenkins et al., 1997). All phases of dye permeability and impermeability are illustrated in color micrographs (Anguish and Ghiorse, 1997). Viable oocysts were the sum of 4′,6-diamidino-2-phenylindole-negative (DAPI − ) propidium iodide-negative (PI − ) oocysts and DAPI + PI − oocysts; DAPI + PI + oocysts were considered inactivated. Empty oocyst shells were also counted as inactivated in this assay.

### 2.6. Microscopy

All samples were examined on a Zeiss LSM-210 microscope as described by Anguish and Ghiorse (1997) and Jenkins et al. (1997). Determination of oocyst inoculum density was performed using a hemacytometer and a Nikon Plan 20×/0.4 objective on a Nikon Labophot-2 with 10× eye-pieces.

### 2.7. Data analysis

Coefficients of inactivation, $K$, for each treatment were determined by using the first-order decay model, $P_t = P_o\, e^{Kt}$, where $P_o$ was the initial percentage of viable oocysts and $P_t$ was the percentage of viable oocysts at time $t$ in days. $K$-values were determined by regressing $\ln(P_o/P_t)$ against $t$. Each regression was tested for linearity and slope using a one-way analysis of variance as described by Zar (1999). Using analysis of covariance and the Tukey's test for multiple comparisons among slopes (Zar, 1999) hypotheses 1–4 were tested. Days to reach 99.9% inactivation were calculated using the equation $t = \ln(P_o/P_t)/K$. All other statistical analyses of data were performed with Minitab statistical software, and the probability value of at least 0.05 was used to determine significant differences between treatments.

## 3. Results

### 3.1. Effects of soil water potential on oocyst inactivation rates (testing hypothesis 1)

Testing hypothesis 1 indicated that at 4 °C for each soil type there was no significant difference in inactivation rates ($K$-values) between the different soil water potentials (Table 2). The $K$-values for oocysts inoculated in the silt loam soil at 4 °C at all three soil water potentials were not significantly different from zero inactivation or zero slope. No differences in rates of inactivation were observed between the three soil water potentials for oocysts incubated in the silty clay loam at 20 °C, and the loamy sand soil at 20 and 30 °C. The oocyst inactivation rates associated with the silty clay loam at 30 °C and −0.5 and −1.5 MPa, and with the silt loam at 20 °C and −0.033 and −1.5 MPa also were not significantly different from one another. Because these data established a trend that indicated no significant differences in $K$-values between the three soil water potentials, the assumption may be made that the missing values (because too few oocysts were recovered for analysis) for the silty clay loam at 30 °C and −0.033 MPa, and the silt loam soil at 20 °C and −0.5 MPa, and at 30 °C and −0.5 and −1.5 MPa conformed to this trend and would not be significantly different from the $K$-values associated with the other soil water potentials at their respective temperatures. The null hypothesis for hypothesis 1 was, therefore, not rejected, and the data for the three soil water potentials at each temperature and soil type were grouped together for regression (Table 3), and plotting survival curves (Fig. 1).

Table 2
Inactivation rates, $K-$values (SE) ($K$-values (SE) for each temperature regime per soil type that are followed by the same letter are not significantly different at $P = 0.05$ by Tukey's test for multiple comparisons among slopes) and coefficients of determination, $r^2$ (*significant at $P = 0.05$, **significant at $P = 0.01$, ***significant at $P = 0.001$)

| Soil type | Temperature (°C) | Soil moisture (MPa) | $K$ (day$^{-1}$) | $r^2$ |
|---|---|---|---|---|
| Silty clay loam | 4 | −0.033 | 0.0016 (0.0003)a | 0.191ns[a] |
| | | −0.5 | 0.0031 (0.0009)a | 0.570* |
| | | −1.5 | 0.0038 (0.0013)a | 0.641* |
| | 20 | −0.033 | 0.0086 (0.0025)a | 0.653* |
| | | −0.5 | 0.0090 (0.0027)a | 0.560* |
| | | −1.5 | 0.0143 (0.0033)a | 0.696* |
| | 30 | −0.033 | ID[b] | |
| | | −0.5 | 0.0259 (0.0015)a | 0.975*** |
| | | −1.5 | 0.0175 (0.0045)a | 0.751* |
| Silt loam | 4 | −0.033 | 0.0014 (0.0010)a | 0.144ns |
| | | −0.5 | 0.0007 (0.0009)a | 0.104ns |
| | | −1.5 | 0.0008 (0.0010)a | 0.117ns |
| | 20 | −0.033 | 0.0029 (0.0010)a | 0.400* |
| | | −0.5 | ID | |
| | | −1.5 | 0.0038 (0.0003)a | 0.671* |
| | 30 | −0.033 | 0.0062 (0.0006) | 0.654*** |
| | | −0.5 | ID | |
| | | −1.5 | ID | |
| Loamy sand | 4 | −0.033 | 0.0032 (0.0025)a | 0.111ns |
| | | −0.5 | 0.0020 (0.0008)a | 0.329* |
| | | −1.5 | 0.0026 (0.0021)a | 0.511* |
| | 20 | −0.033 | 0.0089 (0.0018)a | 0.687*** |
| | | −0.5 | 0.0082 (0.0016)a | 0.703*** |
| | | −1.5 | 0.0108 (0.0026)a | 0.566** |
| | 30 | −0.033 | 0.0091 (0.0016)a | 0.894** |
| | | −0.5 | 0.0116 (0.0037)a | 0.500* |
| | | −1.5 | 0.0115 (0.0013)a | 0.617* |

[a] Not significant.
[b] Insufficient data.

Table 3
Oocyst inactivation rates, $K$-values ($K$-values within each soil type and water control followed by a different letter not in parentheses are significantly different at $P = 0.05$ by Tukey's test for multiple comparisons among slopes, and different letters in parentheses indicate significant differences in $K$-values between soil types and the water at each respective temperature at $P = 0.05$ by Tukey's test for multiple comparisons among slopes), based on pooling of soil moisture data for each soil type at 4, 20 and 30 °C with coefficients of determination, $r^2$, and days to reach 99.9% inactivation (***significant at $P = 0.001$)

| Soil type | Temperature (°C) | $K$-values (day$^{-1}$) | $r^2$ | Days to reach 99% inactivation |
|---|---|---|---|---|
| Silty clay loam | 4 | 0.0030b, (b) | 0.433*** | 2302 |
| | 20 | 0.0111a, (b) | 0.659*** | 622 |
| | 30 | 0.0205a, (b) | 0.826*** | 336 |
| Silt loam | 4 | 0.0012b, (c) | 0.247ns[a] | 4063 |
| | 20 | 0.0030ab, (c) | 0.470*** | 2302 |
| | 30 | 0.0062a, (d) | 0.962*** | 1096 |
| Loamy sand | 4 | 0.0031b, (b) | 0.449*** | 2228 |
| | 20 | 0.0100a, (b) | 0.719*** | 690 |
| | 30 | 0.0109a, (c) | 0.676*** | 634 |
| Water | 4 | 0.0077c, (a) | 0.903*** | 895 |
| | 20 | 0.0298b, (a) | 0.931*** | 231 |
| | 30 | 0.0327a, (a) | 0.935*** | 211 |

[a] Not significant.

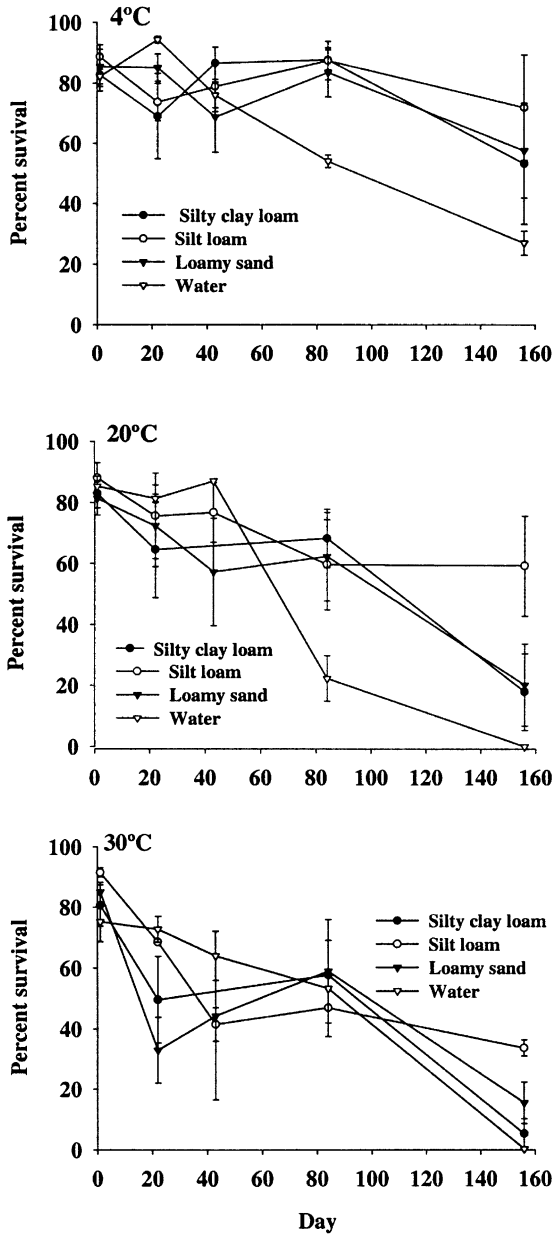


Fig. 1. Oocyst survival curves based on grouped soil water potential data (mean percentage sum of $DAPI^-$ $PI^-$ and $DAPI^+$ $PI^-$ ± SE) for each incubation temperature and soil type. Oocyst survival curves for the temperature controls in water are included.

### 3.2. Effects of soil temperature on oocyst inactivation (testing hypothesis 2)

Based on Tukey's test (Zar, 1999) of the grouped data significant differences in rates of inactivation for oocysts incubated in the silty clay loam and loamy sand were observed between 4 °C and the higher temperatures (Table 3). The oocyst inactivation rates associated with all three soils at 20 and 30 °C were, however, not significantly different from one another, though, with the exception of the oocysts in the silt loam at 20 °C, they were both significantly greater than the inactivation rate at 4 °C (Table 3). As expected the general trend observed was that as the temperature increased, rates of oocyst inactivation significantly increased. Because oocyst inactivation rates at 4 °C were significantly different from the rates associated with 20 and 30 °C, the null hypothesis for hypothesis 2 was rejected.

### 3.3. Effect of soil type on oocyst inactivation (testing hypothesis 3)

The Tukey's test (Zar, 1999) on oocyst inactivation rates between soil types based on the grouped data indicated no differences at 4 °C between the silty clay loam and loamy sand both of which were significantly greater than the *K*-values associated with the silt loam (Table 3). The oocyst inactivation rates at 4 °C in the silty clay loam and loamy sand were greater than zero as indicated by their significant coefficients of determination. There were, however, differences at the higher temperatures. The inactivation rate of oocysts incubated in the silt loam at 20 °C was significantly less than the inactivation rates for oocysts in the silty clay loam and sandy loam (Table 3). At 30 °C oocyst inactivation rates were significantly different between the three soil types; the rate of inactivation of oocysts in the silty clay loam incubated at 30 °C was greater than the other two soil types. Because of the differences in oocyst inactivation rates between soil types at the three incubation temperatures the null hypothesis of hypothesis 3 was rejected.

### 3.4. Rates of oocyst inactivation in soil compared to water controls (testing hypothesis 4)

As expected comparison of regression equations among the temperature controls indicated significant differences between rates of oocyst inactivation. Rates of inactivation increased with increase in temperature (Table 3). The Tukey's test indicated differences in inactivation rates between oocysts incubated in water (temperature controls) and oocysts incubated in the three soil types. In every case, oocyst inactivation rates were significantly less in the different soil types than the inactivation rates associated with the temperature controls. The null hypothesis of hypothesis 4 that no differences between oocysts in the soils and water would be observed was rejected.

### 3.5. Oocyst viability at 156 days

The mean percent viability at 156 days (Table 4) reflects the differences in inactivation rates between soil treatments and water controls. The high percentage of potentially infective oocysts remaining after 156 days in the silt loam soil incubated at 4 °C reflected an inactivation rate not significantly different from zero (Table 3). In contrast, the decline in percent of potentially infective oocysts incubated at 4 °C in the silty clay loam and loamy sand soils at day 156 reflected inactivation rates that were slight but significantly greater than zero (Table 3, and Fig. 1). As the incubation temperature increased the percent of potentially infective

Table 4
Mean percent viability of oocysts (means followed by different letters are significantly different at $P < 0.05$) in the three soil types at day 156 per temperature

| Temperature (°C) | Soil type | | | |
|---|---|---|---|---|
| | Silty clay loam (%) | Silt loam (%) | Loamy sand (%) | Water (%) |
| 4 | 53.4ab | 72.0a | 57.7a | 27.0b |
| 20 | 17.7c | 59.2a | 20.2bc | 0.0e |
| 30 | 5.3 | ID[a] | 12.6cd | 0.3d |

[a] Insufficient data.

oocysts remaining after 156 days significantly decreased. The general trend was for oocysts to persist longer in the silt loam than in either of the other two soil types, and least of all in distilled water.

### 3.6. Days to reach 99.9% inactivation

Based on the coefficients of inactivation (Table 3) the calculated days to reach 99.9% inactivation (Jenkins et al., 1998) indicated that survival at 4 °C in the silt loam at soil water potentials between −0.033 and −1.5 MPa was nearly twice that in either the silty clay loam and loamy sand. Survival in the three soil types appeared to be three times longer than in distilled water. Projected oocyst survival in the silt loam was nearly twice or greater than in the other two soil types at 20 and 30 °C. At all temperatures projected survival of oocysts was greater in all three soil types than in distilled water.

## 4. Discussion

### 4.1. Rates of oocyst inactivation in soil and water

To the best of our knowledge this is the first study of the interactions between different soil types, soil water potentials, and temperatures on the inactivation dynamics of *C. parvum* oocysts. Several studies have been reported on the effect of temperature on inactivation of oocysts suspended in water (Fayer, 1994; Fayer and Nerad, 1996; Fayer et al., 1998; Jenkins et al., 1997, 1999; Olson et al., 1999). Their studies indicated a general pattern that extreme temperatures, $< -5$ and $>35$ °C, accelerated inactivation, and complete inactivation could occur in minutes and hours. For temperatures between 4 and 30 °C, however, rates of inactivation were in terms of weeks and months. The water controls in our study reflected this pattern, and corresponded to results reported by Fayer et al. (1998).

Studies on oocyst survival in soil are few. Our study indicated that under the experimental conditions soil water potentials between −0.033 and −1.5 MPa had little or no effect on oocyst inactivation at any of the temperatures and soil types examined. In contrast to our results, Jenkins et al. (1999) reported increased oocyst inactivation rates with increased negative water potentials that ranged from −0.1 to −3.2 MPa; these rates were associated with a silt loam contained in microcentrifuge tubes. Exposure to bulk soil as in our study may have mediated the effects of soil water potential on oocyst survival. Soil water potentials more negative than −1.5 MPa may still adversely affect oocyst survival (Jenkins et al., 1999).

At temperatures between 4 and 30 °C, rates of oocyst inactivation increased with increased temperature, and followed the pattern that Fayer et al. (1998) observed. Oocyst inactivation was, however, significantly lower in the three field soils than in water. In contrast Olson et al. (1999), who examined oocyst inactivation in distilled water and a field soil (whose particle size distribution of 27.2% clay, 58.2% silt, and 14.6 % sand would classify it as a silt loam (Shirazi et al., 1988)) at 4 and 25 °C and 17% water content, reported greater inactivation in soil than in distilled water.

Our results indicated that between the three soil types there were significant differences in inactivation rates as well as the proportion of oocysts remaining potentially infective at the final sampling date. Greatest oocyst survival was associated with the silt loam at 4 °C. Oocyst inactivation was greatest in the silty clay loam at 30 °C. At all temperatures the oocyst inactivation rates associated with the silty clay loam and loamy sand were significantly greater than the rates associated with the silt loam. The lower pH values of the silty clay loam and loamy sand (Table 1) may appear to be a contributing factor to this difference. The oocyst inactivation rates at 20 °C for the silty clay loam and loamy sand were greater than that of the silt loam; at 30 °C the inactivation rate of the more acidic silty clay loam was significantly different from the loamy sand. Campbell et al. (1992) reported that a 1 h exposure to 0.1 M HCl (~pH 1) inactivated a significant fraction of suspended oocysts; but an HBSS solution acidified to pH 2.75 had no effect on oocyst inactivation. The likelihood, therefore, is not great that the pH of these soils was a factor in the differences in oocyst inactivation rates between the soil types we examined. Furthermore, at temperatures a few degrees above freezing such as 4 °C soil type may be an insignificant factor on oocyst survival.

The rates of inactivation for oocysts suspended in water at 4 °C was less than that reported by Jenkins et al. (1997); whereas, the rate at 20 °C was similar to that reported by Jenkins et al. (1997). The days to reach 99.9% inactivation in the soils were an order of magnitude greater (Table 3)

than that for oocysts in water. These data (under the conditions of the study) indicate that oocysts may survive several months, even a few years in agricultural soils.

### *4.2. Longevity of oocyst survival and its impact on agricultural management*

Infective sporozoites within the oocyst are metabolically active, and are known to use endogenous reserves of amylopectin to sustain their infectivity while in the environment (Fayer et al., 1998). Most likely the reserves of amylopectin that are associated with the sporozoites and oocyst residuum represent the limit of oocyst infectivity (Fayer et al., 1998). Based on the data Fayer et al. (1998) reported regarding the quantity of amylopectin stored in oocysts over time at various temperatures, the days to reach a limit of reserves was calculated to be 465, 308 and 276 days for oocysts exposed to 5, 20 and 30 °C in water, respectively. Using dye permeability and in vitro excystation methods for determining potential oocyst infectivity, Jenkins et al. (1997) reported the presence of potentially infective oocysts in calf manure naturally contaminated with *C. parvum* oocysts after 410 days at 4 °C, and the presence of potentially infective oocysts in distilled water with antibiotics after 667 days at 4 °C. The inactivation rates we report may represent the extreme limits of oocyst survival in watershed soil environments under controlled laboratory conditions. Further research including additional field studies, and studies on the depletion of amylopectin reserves are needed to determine the longevity of oocyst infectivity. From the perspective of agricultural management our results suggest that manure containing *C. parvum* oocysts, such as from neonatal calves, may be applied when soils are warm (around 20 °C or greater). During the cold months of the year calf wastes should be stored in a pile (Jenkins et al., 1999), and then spread on fields during the warmer months of the year.

### *4.3. Conclusions*

Independent of soil texture, soil water potential between −0.033 and −1.5 MPa had an insignificant effect on oocyst survival. Oocyst rates of inactivation were significantly less in the silt loam than the silty clay loam and loamy sand, but temperature appeared to be the factor most affecting oocyst survival. Under constant laboratory conditions survival in soil was significantly greater than in water. Based on our results soils on which animal waste containing *C. parvum* oocysts is applied may be considered a long-term reservoir of infective oocysts, and under certain conditions of climate and landscape may threaten to pollute surface waters of watersheds with this zoonotic pathogen.

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